Soluble Mediators Released by Flow- and Pressure-Exposed Vascular Endothelial Cells Induce Functional Changes in Endothelial and Smooth Muscle Cells

Jennifer A. McCann, Thomas J. Webster, and Karen M. Haberstroh
Department of Biomedical Engineering
Purdue University
West Lafayette, IN

Introduction. The two most important cell types in the arteries are endothelial and smooth muscle cells; the behavior of these cells is determined by both hemodynamic and biochemical factors. In particular, shear stress and pressure forces have a direct impact on vascular endothelial and smooth muscle cell morphology and phenotype. In addition to direct hemodynamic effects, the communication system between these two cell types also participates in vessel homeostasis and the development of diseases like atherosclerosis. This cell communication occurs due to the matrix-like structure of the arterial wall, which allows for chemical mediators released from one cell type to affect the functions of neighboring vascular cells. Based on this knowledge, the present study investigated vascular endothelial and smooth muscle cell responses to medium conditioned by cells exposed to either shear stress or pressure forces.

Materials and Methods.

Exposure of Rat Aortic Endothelial Cells (RAECs) to Hemodynamic Forces. RAECs were seeded (35,000 cells/cm^2) onto etched glass cover slides previously coated with fibronectin. Once confluent, these cells were exposed either to laminar fluid flow (τ = 10.4 dynes/cm^2) in a parallel plate flow chamber or to a physiological cyclic pressure stimulus (max = 120 mmHg, min = 70 mmHg, freq = 1 Hz) in a pressurized gas environment for 6 hours. Upon completion of each experiment, the supernatant medium was collected from RAECs. *Flow-conditioned* medium contained any soluble and transferable factors released by RAECs in response to laminar fluid flow. Similarly, *pressure-conditioned* medium contained any factors released in response to pressure. Finally, *control-conditioned* medium contained any factors released by RAECs under standard cell culture conditions.

RAEC mRNA Expression Following Exposure to *Conditioned* Medium. RAECs were seeded (35,000 cells/cm^2) onto fibronectin-coated, etched glass and incubated under standard cell culture conditions in MCDB-131 medium. After 24 hours, the standard medium was removed and RAECs were subjected to the following conditions for 6 hours: control medium (MCDB-131) in a static environment, control medium and laminar fluid flow, control medium in a pressurized environment, *control-conditioned* medium in a static environment, *control-conditioned* medium in a pressurized environment, *flow-conditioned* medium in a static environment, *flow-conditioned* medium and laminar fluid flow, or *flow-conditioned* medium in a pressurized environment. Following each treatment, mRNA expression of ecNOS, COX-2, and PDGF-B by RAECs was determined using RT-PCR.

Vascular Cell Growth Following Exposure to *Conditioned* Medium. RAECs were seeded (1,750 cells/cm^2) as described previously; similarly, Rat Aortic Smooth Muscle Cells (RASMCs) were seeded (1,750 cells/cm^2) onto etched borosilicate glass coverslips and incubated under standard cell culture conditions in DMEM supplemented with 10% FBS and 1% P/S for 24 hours. After 24 hours, the original medium was removed and replaced with fresh MCDB-131 medium (control), *control-conditioned* medium, *flow-conditioned* medium, or *pressure-conditioned* medium. Cells were grown in the presence of these medium types for 1,

3, and 5 days. At each point, adherent cells were fixed and stained, then counted, averaged, and reported as cell density (cell/cm^2).

RASMC mRNA and Protein Expression Following Exposure to *Flow-Conditioned* Medium. RASMCs were seeded (35,000 cells/cm^2) as described above. After 24 hours, the original medium was removed and replaced with control, *control-conditioned*, or *flow-conditioned* medium. RASMCs were allowed to grow in a static environment under standard cell culture conditions in these medium types for 1, 3, and 5 days. At the end of each time period, mRNA expression was analyzed with primers specific to PDGF-AR, iNOS, COX-2, and GAPDH. In addition, protein expression of COX-2 and iNOS was evaluated using fluorescence microscopy after 5 days in culture.

Results and Discussion.

RAEC mRNA Expression was Altered in Response to Hemodynamic Forces and *Conditioned* Medium. Results of this study provide evidence that in response to shear and pressure forces, RAECs release chemical mediators which are able to affect the mRNA expression of other endothelial cells. Specifically, exposure to *control-conditioned* medium alone caused a decrease (p<0.05) in mRNA expression of COX-2, ecNOS, and PDGF-B, while exposure to *flow-conditioned* medium caused the mRNA expression of each molecule to increase (though not always significantly). In addition, increased (p<0.05) mRNA expression was observed under flow alone, which supports previous reports of increased mRNA expression under laminar flow [1-3]. Preliminary data of endothelial cell mRNA expression under the physiological pressure stimulus suggested that the mRNA expression of PDGF-B was slightly increased, while expressions of COX-2 and ecNOS were decreased. However, the most dramatic changes in endothelial cell mRNA expression occurred when hemodynamic forces and *conditioned* medium effects were combined. For instance, the most increased mRNA expressions (p<0.01 for COX-2 and ecNOS and p<0.05 for PDGF-B) occurred when cells were exposed simultaneously to *flow-conditioned* medium and laminar fluid flow.

These results suggest that cells have unique responses to their chemical environment, hemodynamic environment, and the combined effects of both. A similar concept was demonstrated by Zhao et al. [4], who observed that cells exposed to both shear stress and cyclic stretch were more elongated and aligned than cells exposed to either stimulus alone. Together, these results demonstrate the importance of isolating the effects of one component in a system, while underscoring the important fact that results may change when such factors are presented in combination.

Vascular Cell Growth was Altered in Response to *Conditioned* Medium. The effects of biochemical stimulation alone were further investigated by monitoring vascular cell growth patterns in response to *conditioned* medium. Results of this study provided evidence of decreased RAEC growth under both *flow-* and *pressure-conditioned* medium, while cells grown under *control-conditioned* medium had increased (p<0.01 on days 1 and 3 compared to controls, p<0.01 on days 3 and 5 compared to *flow-conditioned* samples, and p<0.05 at each time point compared to *pressure-conditioned* cells) cell growth. In contrast, RASMC growth was increased (p<0.05 compared to control and *control-conditioned* samples) at each time point following exposure to both *flow-*and *pressure-conditioned* medium. Interestingly, RASMC growth under control and *control-conditioned* medium was similar at each time point.

In contrast to the present findings, previous results suggested that endothelial cell growth was similar under all medium types [5], while smooth muscle cells responded to *control-conditioned* [5,6] and *flow-conditioned* [5] medium with increased cell growth when compared to controls. In contrast, the present findings demonstrated media and cell type

specific responses. Namely, smooth muscle cell growth was significantly enhanced under *flow-conditioned* medium, while endothelial cell growth was inhibited in the presence of *flow-conditioned* medium and enhanced in the presence of *control-conditioned* medium. These study differences may be due to medium storage effects, as both Ono et al [5] and Peiro et al [6] stored their medium at low temperatures prior to use. Finally, previous studies by Vouyouka et al [7,8] utilized *pressure-conditioned* medium and found decreased endothelial cell growth only in response to medium conditioned under cyclic pressures (160/100 mmHg), while smooth muscle cell growth was decreased in response to medium conditioned under static pressures (135 mmHg). A potential explanation for the cell-specific responses to both *flow-* and *pressure-conditioned* medium observed in the present study may be the composition of these media, as each cell type is responsive to specific mediators. For example, smooth muscle cell growth is significantly impacted by the presence of PDGF, while endothelial cells are not responsive to this mediator.

RASMC mRNA and Protein Expression was Increased in Response to *Flow-Conditioned* Medium. Mediators released by flow-exposed RAECs altered the mRNA expression of PDGF-AR, iNOS, and COX-2 in RASMCs. Specifically, PDGF-AR expression in *flow-conditioned* samples was down regulated ($p<0.05$) on day 1, but up regulated ($p<0.05$) on days 3 and 5, with its maximum expression occurring on day 3. Similarly, mRNA expression of iNOS was induced ($p<0.05$ on days 3 and 5) only in cells exposed to *flow-conditioned* medium and had maximal expression on day 3. Finally, COX-2 mRNA expression also followed a bell shaped trend; it was up regulated ($p<0.01$) at each time point. These findings were the consequence of flow-induced mediators alone since mRNA expression by cells exposed to *control-conditioned* medium did not differ from that of control cells. Increased mRNA expression of these mediators may indicate their roles in transduction pathways ultimately leading to increased RASMC growth under *flow-conditioned* medium. In support of this mRNA data, quantitative protein expression studies demonstrated that exposure of RASMCs to *flow-conditioned* medium led to increased (though not specifically localized) iNOS and COX-2 protein expression when compared to controls.

Conclusions. Overall, results of this study demonstrate the importance of studying cell responses to individual stimuli, while also emphasizing the fact that cell responses will differ in an environment more closely resembling the physiological state. Ultimately, studies like these will contribute to an understanding of vascular cell communications, and may aid in the development of drug therapies for vascular diseases like atherosclerosis.

Acknowledgements. The authors would like to thank the Showalter Foundation (Award # 1220012825), the Purdue University Special Initiatives Fellowship, and the Purdue Research Foundation Research Grant (Award # B905S) for assistance in funding this project, and Dr. Fred Pavalko (IUPUI) for supplying the flow apparatus.

References. [1] Topper, J.N., et al. Proc. Natl. Acad. Sci., 1996; 93: 10417-10422. [2] N. Resnick, et al. Proc. Natl. Acad. Sci., 1993; 90: 4591-4595. [3] D. M. Lloyd-Jones and K.D. Bloch. Ann. Rev. Med., 1996; 47: 365-375. [4] S. Zhao, et al. Arterioscler. Thromb. Vasc. Biol., 1995; 15: 1781-1786. [5] O. Ono, et al. Cell Struct. Funct., 1991; 16: 365-374. [6] C. Peiro, et al. Hypertension, 1995; 25: 748-751. [7] A. G. Vouyouka, et al. J. Mol. Cell. Cardiol., 1998; 30: 609-615. [8] A.G. Vouyouka, Y. Jiang, and M.D. Basson. Surgery, 2004; 136: 282-290.